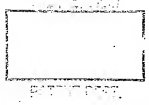
CONFIDENTIAL
INVENTION DISCLOSURE



The invention herein described was made during the course of my employment and is being submitted in pursuance of the terms of the Employee Confidentiality Agreement.
(Please use ink and attach extra sheets of paper if needed.)

1. Title of Invention:

Mutated Epidermal Growth Factor Receptor as a selectable cell surface marker.

2. Brief Description of Invention:

Describe the invention, include any drawings, chemical structures, equipment designs, process steps. Experimental data may be included.

The present invention provides a method to use mutated versions of the epidermal growth factor receptor (EGFR) as a selectable cell surface marker. The EGFR was mutated in the extracellular as well as the intracellar domain in such a way that neither ligand binding nor signal transduction through this receptor occurs (see Fig.1 mutated EGFRII). This will therefore render the molecule inert. Thus, introduction of this mutated EGFR in eukaryotic cells e.g. cells of hematopoietic or others should provide a safe means to identify and select mutated EGFR expressing cells with an antibody directed against the mutated EGFR. Other molecules that were similarly rendered inert by mutating the intracellular and extracellular domain include Muscle specific receptor receptor tyrosine kinase (MuSK) or the γ-amino butyric acid receptor $_B$1/2 (GABAR$_B$1/2). These mutated molecules can be also used as selectable marker.

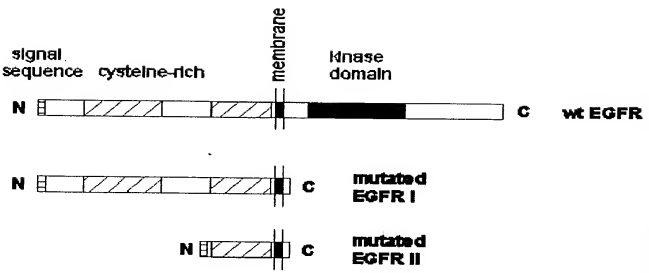


Fig.1.: This figure shows the mutations that were introduced in the EGFR to render the molecule inert. EGFRI has a mutation/deletion in the intracellular domain only. EGFRII has an additional mutation/deletion in the extracellular domain.

3. Novel Aspects:

Describe novel aspects of invention, i.e., how it is new and different.

The invention is new in that human cell surface molecules e.g. EGFR, MuSK, $GABAR_g1/2$ are used, which have not been used in the past for this kind of application.
Previously, cell surface molecules that were used as cell surface markers were mutated in their intracellular domain to avoid signaling of these newly introduced molecules when they would bind to their ligand. However, upon binding of to their ligands these intracellularly mutated molecules could potentially still heterodimerize with endogenous receptors and could therefore result in a dominant negative effect (see Fig.1/mutated EGFRI. This molecule has been previously described in patent WO 93/05148 as a mutant EGFR that is called HERCD-533 and that is devoid of signaling activity.). To avoid this problem we also mutated parts of the extracellular domain to prevent ligand binding (see Fig. 1 mutated EGFRII). However, extracellular mutations were done in such a way that antibody binding to the extracellular domain can still occur and therefore effective identification and selection of marker gene carrying cells is possible. This therefore adds another new safety feature to the usage of these molecules as cell surface markers.

4. Pertinent References of Which You are Aware:

List literature (including abstracts), patent applications, patents, and presentations, with respect to efforts to deal with the kind of problem your invention is designed to solve.

O. Kashles, Y. Yarden, R. Fischer, A. Ullrich and J. Schlessinger MCB 1991, 11: 1454-1463, A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization.

C. R. Lin, W. S. Chen, W. Kruiger, L. S. Stolarsky, W. Weber, R. M. Evans, I. M. Verma, G. N. Gill, M. G. Rosenfeld. Science 1984, 224: 843-847: Expression Cloning of Human EGF Receptor Complementary DNA: Gene Amplification and Three Related Messenger RNA in A431 Cells.

Human epidermal growth factor receptor cDNA is homologous to a variety of RNAs overproduced in A431 carcinoma cells.

K. Kaupmann, K. Huggel, J. Heid, P. J. Fior, S. Bischoff, S. J. Mickel, G. McMaster, C. Angst, H. Bittiger, W. Froestl, B. Bettler. Nature 1997, 386: 239-246. Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors.

K. Kaupmann, B. Malitscheck, V. Schuler, J. Heid, W. Froestl, P. Beck, J. Mosbacher, S. Bischoff, A. Kulik, R. Shigemoto, A. Karschin, B. Bettler. Nature 1998, 396: 683-687. $GABA_B$-receptor subtypes assemble into functional heteromeric complexes.

D.M. Valenzuela, T. N. Stitt, P. S. DiStefano, E. Rojas, K. Mattsson, D. L. Compton, L. Nunez, J. S. Park, J. L. Stark, D. R. Gies, S. Thomas, M. M. LeBeau, A. A. Fernald, N. G. Copeland, N. A. Jenkins, S. J. Burden, D. J. Glass, G. Yancopoulos. Neuron 1995, 15: 573-584. Receptor tyrosine kinase specific for the skeletal muscle lineage: Expression in embryonic muscle, at the neuromuscular junction, and after injury.

Patents: WO93/05148, PCT/EP94/02687

5. Utility of Invention:

Describe any other possible applications of the invention beyond the intended primary application. Describe any commercial aspects of the invention.

The selectable marker will be part of a product, either cell e.g. hemopoietic stem cell or vector system. Thus the commercial value will depend on the product the selectable marker is sold with.

6. Date of Invention:

7. Disclosure Outside of SyStemix:

List places, dates and names of persons or companies to whom disclosed (or planned to be disclosed) outside of SyStemix (regardless of the existence of a nondisclosure agreement).

N/A

8. Documentation:

A notebook reference and location of notebook.

See attached documents

9. Program or Contract:

Was invention made during the course of your work on a specific program or contract?

Yes: ___x_____ No: _____ Specific program or contract: ___34/05_____

10. Persons who Worked on Invention:

Susanne Pippig
Peter Chang
Gabor Veres

11. Person Preparing this Disclosure:

Signature: _____ Printed name: Susanne Pippig

Address: ___Systemix, Inc. 3155 Porter Drive, Palo Alto, CA 94304____

Date: _____ _____

12. Two Witnesses:

The invention was described to me by the above inventor(s); the description was examined and clearly understood.

Signature: _____ Printed name: ____Fernando Rock_____

Address: ___Systemix, Inc. 3155 Porter Drive, Palo Alto, CA 94304____

Date: ___ _____

Signature: _____ Printed name: ____Ann Marie O'Farrell____

Address: ___Systemix, Inc. 3155 Porter Drive, Palo Alto, CA 94304____

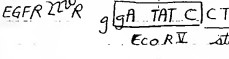
Date: ___ _____

Purpose: clone hEGFR deletion mutant

A) hEGFR PCR

Order primers from Operon

		Tm
hEGFR186F	cc**T CTA gA**g ATg CgA CCC TCC ggg Acg (**x6a1**)	~68.8°C
hEGFR2220R	g**gA TAT C**CT ACg TgC gCT TCC gAA CgA TgT g (**thr654**; **EcoRV** stop codon)	~66.5°C



		5' 94°C
1 μg/λ pNTK-EGFR	0.2 λ	↓
hEGFR2220R (Tm 71°C)	1	94°C 30"
hEGFR186F (Tm 74°C)	1	66°C 60" (30×)
pfu buffer	5	72°C 5'
10mM dNTP	2	↓
dd H2O	38.8	72°C 10'
pfu	2	↓
	50λ	4°C

		10X
hEGFR PCR from above	2 λ	20
hEGFR2220R	1	10
hEGFR186F	1	10
pfu buffer	5	50
10mM dNTP	2	20
dd H2O	39	380
pfu	1	10
	50λ	50λ/tube

94°C 5' → (94°C 30" / 68°C 60" / 72°C 6') 10X → 72°C 10' → 4°C soak

load 12 λ from 450λ PCR product (pg 66)

Gel Isol.

PCR Scr. Ligation → Tfm

Sets Susanne to take out plates & Innoculate

Take out late ⇒ satelite colonies MUSK H2 4 H3

~~Put into mini-prep~~

EGFR

EcoRS + Stu I

Innoculate # 2 & 5

?

Qiagen maxiprep ⇒ pca yield?

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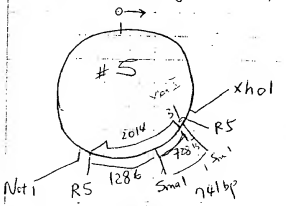
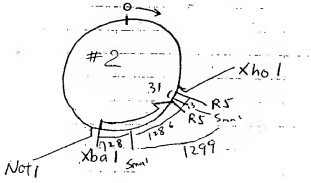
H2 H3

Signed Date Signed Date

[redacted] - Inoculate. Also streak - check maxiprep (pg 66)

[redacted] Qiagen maxiprep again





EcoRS	2045, 2930 bp ✓	
Xba I	4944 bp ✓	
Sma I	741, 4257 bp	didn't cut ?

pCRScn-hEGFR #5

728, 4216 bp didn't cut

4944

1299, 3689 bp star activity ? ? ✓

pCRScn-hEGFR #2

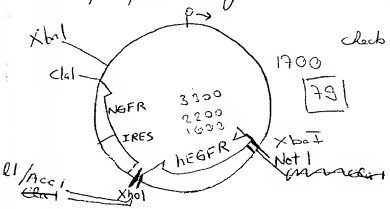
) clone into pG1a-IRES-NGFR ∴ pG1a-hEGFR-IRES-NGFR

[redacted]

#1210 pG1a-IRES-NGFR (0.8 μg/λ)	12.5	pCRScn-hEGFR #2	25
Buffer H	10	Buffer H	10
Not I	5	Not I	5
Xho I	5	Xho I	5
TE	57.5	TE	55
	100 λ		100 λ

37°C 2:45

ethanol precipitate → gel isolation → Rapid DNA ligation kit : 1.5λ #1210
6.5λ pCRScn-hEGFR #2



clock miniprep w/ Xba I

1700

79

40λ

Tfm XL 10 gold

w/ 2λ Ligt

Continued on Page 72

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Peter Chang
Signed

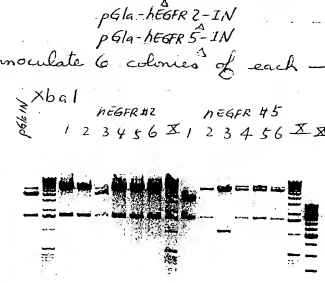
[redacted]
Date

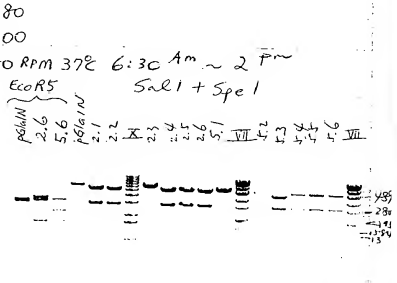
Jennifer Lu
Signed

[redacted]
Date

	# colonies	
[redacted] #1210 control	12	plasmid
hEGFR2△ insert control	0	
hEGFR5△ insert control	0	
pGla-hEGFR2△-IN	180	
pGla-hEGFR5△-IN	200	

innoculate 6 colonies of each → 250 RPM 37℃ 6:30 AM ~ 2 PM





XbaI in primers methylation sensitive?

	pGla IN	pGla-hEGFR-IN
EcoR5	75, 76, 3016, 3025	32, 75, 76, 1750, 3016, 3330
SalI+SpeI		2868, 5408

[redacted] Qiagen maxiprep

(C) clone pGla-hEGFR [81]

[redacted]

pGla	22 λ	0.452 μg/λ
H	10	
NotI	5	
XhoI	5	
TE	78	
	100 λ	37℃ 10:10 AM – 12:40 PM

dephosphorylation: add to above 100 λ reaction

20 λ CIP buffer	
8 λ CIP	
72 λ TE	
200 λ	37℃ 1 PM ~

⇒ ethanol precipitate

(D) clone pGla-IRES-hEGFR?

eluted pGla (NotI+XhoI)	5040
buffer L	5
10mM dNTP	3
Klneow	2
	50 λ

30℃ 15′
75℃ 15′

Repeat above for pCRSu-hEGFR #2 frgmt (NotI+XhoI)

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[redacted]
Date

[redacted]
Signed

[redacted]
Date

Ⓒ gel isolation → elute in ~~100~~ 80 λ ~100ng/λ

rapid DNA ligation

pGla (NotI+XhoI)	1
pCRScr-hEGFRΔ #2 (NotI+XhoI)	7
buffer 2	2
" 1	10
T4 DNA ligase	1
	21

empty vector
1 4λ pGla NotI+XhoI, Klnw fragmt A } gel
2 4λ pGla " " " fragmt B } isol
3 4λ pCRScr-hEGFRΔ #2 " " "
4 4λ IRES XhoI+NcoI, Klnw

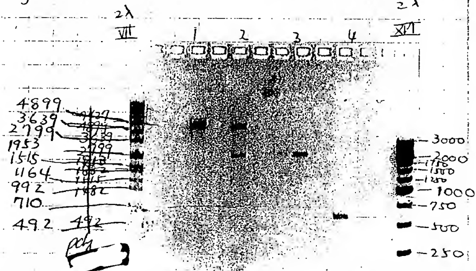
Ⓓ rapid DNA ligation (3 way ligation)

pGla (NotI+XhoI, Klnw)		1
pCRScr-hEGFRΔ (NotI+XhoI, Klnw)	4	9
IRES (XhoI+NcoI, Klnw)	3	6
buffer 2	2	4
" 1	10	20
T4 DNA ligase	1	2
	21 λ	42 λ

25°C overnight pcl [redacted]

[redacted]

tfm XL1 Blue (125λ) w/ 4λ
ligation mixture → 30' ice
42°C 90"
ice 2'
37°C 250 RPM 30'
plate → 37°C shaker 1 pm



4λ loaded. 1% TEA agarose

[redacted]

innoculate 6 colonies from Ⓒ #1-6 } ~7 Am
12 Ⓓ 7-18 } ~250 RPM 37°C →

	# colonies	check w/ SmaI	XbaI	NaeI + SpeI
pGla Φ Ⓒ	1 2 0	1580, 3093	—	4673
pGla-hEGFR Ⓒ	1 2 0 0	~~1997~~, 584, 749, 1015, 1324, 3093	—	2619, 4146
pGla Φ Ⓓ	2 0 0	1580, 3093	—	
pGla-IRES-hEGFR Ⓓ	1 0 0 0	—	308, 4309	

miniprep
RE 1 λ
buffer 1.2 λ
12 λ
37°C 90'

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Signed Date

[signature] [redacted]
Signed Date

PROJECT hEGFR Δ

Notebook No 1339 23

Continued From Page

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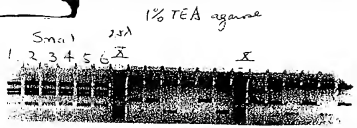
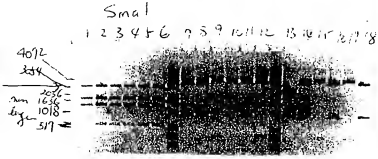




conclusion:

① All 6 colonies of pG1a-hEGFR give expected fragment sizes for Sma I & Nae I + Nae I + Spe I digestion → inefficient in buffer A : Spe I (75-100%).

② 3 way ligations didn't work for pG1a-IRES-hEGFR

Inoculate 5CC mL LB + 100 μg/mL Amp → Qiagen maxiprep for pG1a-hEGFR
Thu 9-17-98 Harvest bacteria & store in -70°C
Fri 9-18-98 Qiagen maxiprep 1 column for 1L → Wash 3x w/ 70% ethanol
get ~ 1500 λ (1 μg/λ)

clerk with map

Peter Chang [redacted] [redacted]

[redacted]

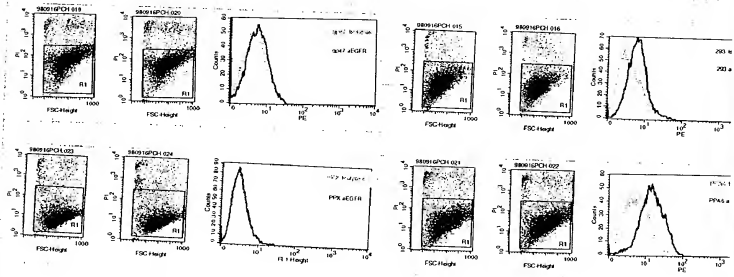
Seed 5-7 x 10^5 293 T's onto a 6 well plate well w/ 2mL
9:15 AM change/replace with fresh DMEM+10% FBS (2mL)
Add 10 λ 10 mM chloroquine to final conc. 50 μM
Put back into incubator ½ - 3 hrs
9:45 AM

				for 10 cm d
3 μg	pCIGL	(1.3 μg/λ)	2.5 λ	15 μg
3 μg	pCIGP	(2 μg/λ)	1.5 λ	15 μg
6 μg	pGIa-hEGFR-IRES-NGFR	(1 μg/λ)	6	30 μg
	H_2O			λ
12.5 λ	2M Ca			60.0 λ
100 μl	in Falcon FACS tubes w/conical bottom			

add drop by drop equal volume of HEPES while vortexing
room temperature 15 ~ 20 min
10:05 AM vortex briefly before adding mixture to cells
mix "∞"
37°C
5:30 PM wash 1x & replace with 2mL fresh DMEM+10% FBS

[redacted]

Spin down cells from sup. Transfect PPA6 WT
Repeat spinoculation on PPA6 WT
Stain with αhEGFR + PE



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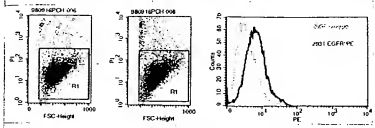
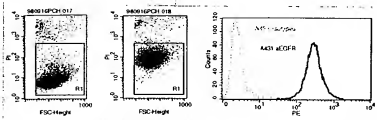


pGlahEGFRIN = correct construct

pGlahEGFRIN56 = wrong orientation /opposite gives no signal, as expected



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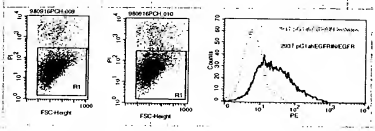
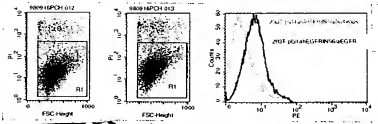
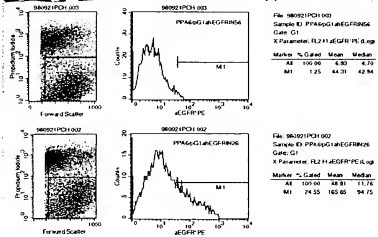




stain PPA6 for NGFR and EGFR



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Date

td FCEMSS WT fwd supernatant pGla hEGFRINGER from PPA6 EPBI's

Abandoned ° cells died

Repeat 1× more → cells still died (from Jennifer Xu)

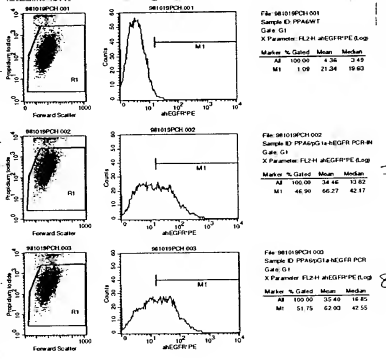
Reseed 293T

tfm

td PPA6 WT from Idem

FACS

SORT for cells



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Signed [redacted] Date

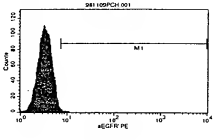
Purpose: Transduce PPAB pGla-hEGFRΔ #79,81 onto CEMSS, CD34+

Transduce CEMSS 2.5 hrs 32°C 3000 RPM

FACS

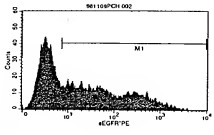
CEMSS

CD34+



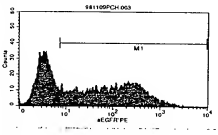
Sample ID: CEMSS/WT
Acquisition Date: 9-Nov-98
Gate: G1

Marker	% Gated	Mean	Median
All	100.00	3.66	3.37
M1	6.92	22.07	8.70



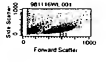
Sample ID: CEMSS/79
Acquisition Date: 9-Nov-98
Gate: G1

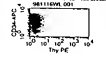
Marker	% Gated	Mean	Median
All	100.00	110.02	10.46
M1	55.68	194.71	52.33

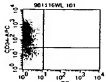


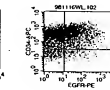
Sample ID: CEMSS/81
Acquisition Date: 9-Nov-98
Gate: G1

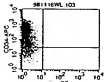
Marker	% Gated	Mean	Median
All	100.00	134.53	20.17
M1	61.63	219.29	102.74

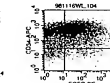


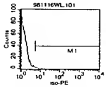


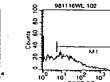


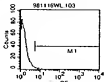


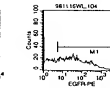












Sample ID: 79 EGFR-PE
Gated Events: 1735
Y Parameter: FL4-H CD34-APC (Log)

Quad	Events	% Gated	X Mean	Y Mean
UL	3617	49.25	4.70	222.87
UR	3300	47.69	82.86	476.71
LL	463	5.99	3.66	16.58
LR	153	1.08	71.34	16.77

Sample ID: 81 EGFR-PE
Gated Events: 7564
Y Parameter: FL4-H CD34-APC (Log)

Quad	Events	% Gated	X Mean	Y Mean
UL	2951	38.91	4.63	443.58
UR	4291	55.28	155.26	594.91
LL	290	3.82	3.44	15.76
UR	142	1.87	178.19	17.67

Sample ID: 79 neo-PE
Gated Events: 7527

Events	% Gated	Mean
7527	100.00	1.37
11	0.15	6.83

Sample ID: 79 EGFR-PE
Gated Events: 7735

Events	% Gated	Mean
7735	100.00	39.27
4919	63.59	60.44

Sample ID: 81 neo-PE
Gated Events: 7607

Events	% Gated	Mean
7607	100.00	1.41
23	0.30	6.90

Sample ID: 81 EGFR-PE
Gated Events: 7564

Events	% Gated	Mean
7564	100.00	51.32
5429	71.58	126.58

P.A.R.P.

Continued on Page 20

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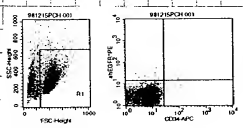
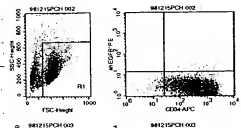


File: 981215PCH001
Sample ID: CD34+/-Isotypes
Gate: G1
X Parameter: FL4-H CD34-APC (Log)
Quad Location: 27, 15

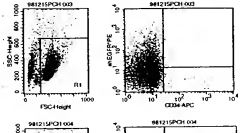
Quad	% Gated	X Mean	Y Mean
UL	0.17	10.76	27.25
UR	0.04	51.92	19.42
LL	99.67	8.94	2.87
LR	0.13	44.52	6.04



File: 981215PCH002
Sample ID: CD34+/aCD34*APC
Gate: G1
X Parameter: FL4-H CD34-APC (Log)
Quad Location: 27, 15

Quad	% Gated	X Mean	Y Mean
UL	0.00	***	***
UR	0.18	727.48	26.53
LL	1.41	17.60	3.62
LR	98.41	769.88	2.04

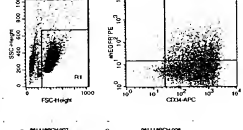
α CD34 APC
1:50 PE



File: 981215PCH003
Sample ID: CD34+/gG1aEGFR-IN/aCD34*AP
Gate: G1
X Parameter: FL4-H CD34-APC (Log)
Quad Location: 27, 15

Quad	% Gated	X Mean	Y Mean
UL	30.58	8.18	63.59
UR	0.14	32.20	152.92
LL	68.80	7.72	8.35
LR	0.38	520.32	4.13

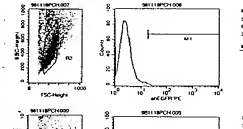
α EGFR PE
1:50 APC



File: 981215PCH004
Sample ID: CD34+/gG1aEGFR-IN/aCD34*AP
Gate: G1
X Parameter: FL4-H CD34-APC (Log)
Quad Location: 27, 15

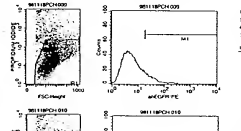
Quad	% Gated	X Mean	Y Mean
UL	0.35	18.50	95.02
UR	27.93	772.56	64.13
LL	2.25	18.25	5.79
LR	70.37	801.24	5.88

α EGFR
CD34



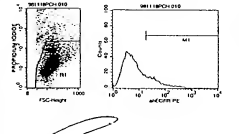
File: 981118PCH008
Sample ID: PBL/Mock
Gate: G3
X Parameter: FL2-H ahEGFR PE (Log)

Marker	% Gated	Mean	Median
All	100.00	3.01	2.48
M1	0.35	21.13	20.17



File: 981118PCH009
Sample ID: PBL/79
Gate: G3
X Parameter: FL2-H ahEGFR PE (Log)

Marker	% Gated	Mean	Median
All	100.00	11.11	5.47
M1	17.99	44.96	30.92



File: 981118PCH010
Sample ID: PBL/81
Gate: G3
X Parameter: FL2-H ahEGFR PE (Log)

Marker	% Gated	Mean	Median
All	100.00	18.57	5.78
M1	15.78	70.15	38.72

Continued on Page 21

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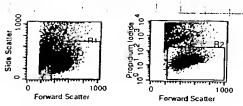
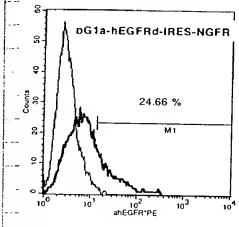
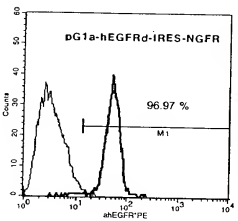
Signed [redacted] Date

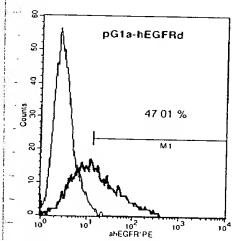


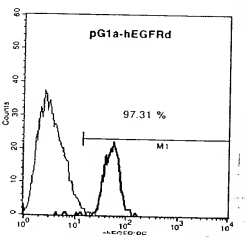
Before Bead Selection

After Bead Selection









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Date

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Date

SYSTEMIX	
Order Number:	033670 01
Order Date:	

GIBCO BRL Custom Primers

Certificate of Analysis

Primer 1:

Primer Name: EGFR1
Researcher: Susanne Pippig
Sequence (5' to 3'): CTA GGC TAG CAT GCG ACC CTC CGG GAC GGC C
Molecular Weight (µg/µmole): 9993.2
Millimolar Extinction Coeff.:(OD/µmol) 320.5

Purity	Standard
Tm (1 M Na+)	89
Tm (50 mM Na+)	67
% GC	70
Notes:	

Primer Number: M0371C12 (C12)
Primer Length: 31
Scale of Synthesis: 50nmol
µg per OD: 31.1
nmoles per OD: 3.1

OD's	31.35
µg's*	977.59
nmoles	97.8
Coupling Eff.	99%

Primer 2:

Primer Name: EGFR2
Researcher: Susanne Pippig
Sequence (5' to 3'): CTC TGC CCG GCG AGT CGG GCT GAC AGC TAT GAG ATG GAG GAA
Molecular Weight (µg/µmole): 13742.4
Millimolar Extinction Coeff.:(OD/µmol) 465.6

Purity	Standard
Tm (1 M Na+)	91
Tm (50 mM Na+)	69
% GC	61
Notes:	

Primer Number: M0371D01 (D01)
Primer Length: 42
Scale of Synthesis: 50nmol
µg per OD: 29.5
nmoles per OD: 2.1

OD's	30.09
µg's*	887.97
nmoles	64.6
Coupling Eff.	99%

Primer 3:

Primer Name: EGFR3
Researcher: Susanne Pippig
Sequence (5' to 3'): TTC CTC CAT CTC ATA GCT GTC AGC CCG ACT CGC CGG GCA GAG
Molecular Weight (µg/µmole): 13484.4
Millimolar Extinction Coeff.:(OD/µmol) 427.2

Purity	Standard
Tm (1 M Na+)	91
Tm (50 mM Na+)	69
% GC	61
Notes:	

Primer Number: M0371D02 (D02)
Primer Length: 42
Scale of Synthesis: 50nmol
µg per OD: 31.5
nmoles per OD: 2.3

OD's	16.66
µg's*	525.99
nmoles	38.9
Coupling Eff.	99%

FOR LABORATORY RESEARCH USE ONLY.

CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

* -See Note about Quantities in Supporting Information.

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SYSTEMIX	
Order Number:	033670 02
Order Date:	

GIBCO BRL Custom Primers

Certificate of Analysis

Primer 1:

Primer Name: EGFR
Researcher: Susanne Pippig
Sequence (5' to 3'): GTT CCT GTG GAT CCA GAG GAG
Molecular Weight (µg/µmole): 6842.2
Millimolar Extinction Coeff.:(OD/µmol) 231.7

Purity	Standard
Tm (1 M Na+)	73
Tm (50 mM Na+)	51
% GC	57

Notes:

Primer Number: Z7143C02 (C02)
Primer Length: 21
Scale of Synthesis: 50nmol

µg per OD: 29.5
nmoles per OD: 4.3

OD's	10.80
µg's*	319.05
nmoles	46.6
Coupling Eff.	99%

Primer 2:

Primer Name: GABA2
Researcher: Susanne Pippig
Sequence (5' to 3'): GGT TCA AGA TCT ACG ACC CTT
Molecular Weight (µg/µmole): 6721.2
Millimolar Extinction Coeff.:(OD/µmol) 223.9

Purity	Standard
Tm (1 M Na+)	69
Tm (50 mM Na+)	47
% GC	47

Notes:

Primer Number: Z7143C03 (C03)
Primer Length: 21
Scale of Synthesis: 50nmol

µg per OD: 30.0
nmoles per OD: 4.4

OD's	9.44
µg's*	283.50
nmoles	42.2
Coupling Eff.	98%

Primer 3:

Primer Name: GABA5
Researcher: Susanne Pippig
Sequence (5' to 3'): CCC TCA CTT ATA AAG CAA ATG
Molecular Weight (µg/µmole): 6698.2
Millimolar Extinction Coeff.:(OD/µmol) 236.9

Purity	Standard
Tm (1 M Na+)	65
Tm (50 mM Na+)	43
% GC	38

Notes:

Primer Number: Z7143C04 (C04)
Primer Length: 21
Scale of Synthesis: 50nmol

µg per OD: 28.2
nmoles per OD: 4.2

OD's	10.99
µg's*	310.76
nmoles	46.3
Coupling Eff.	98%

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CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

* See Note about Quantities in Supporting Information.

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PROJECT Generation of EGFR ΔXC

Notebook No. 1398

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for cytokine prod.

for prolif

\+ PHA + feeder + 40u-IL-2

\+ 20u IL-2 /ml

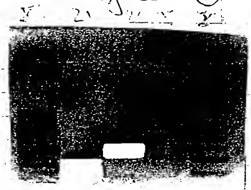
\+ nothing

PCR to generate deletion in the XC domain for the EGFR

(21) ✓ 1l EGFR plasmid (40ng/μl)
✓ 1l EGFR 1
✓ 1l EGFR 3
✓ 2l dNTPs
✓ 10l Pfu buffer
1l Pfu
✓ 84l H_2O
100l

(22) ✓ 1l EGFR pl
✓ l EGFR 2
✓ 1l 4
✓ "
✓ "
✓ "
✓ "
"
100l

30 cycles @ 55°C



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Signed: [illegible] Date: [redacted]

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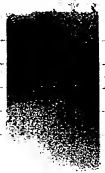
Signed: Marie O'Farrell Date: [redacted]

Hafner
13/14 21/22



23 ✓ 1ℓ EGFR1

	1ℓ	n	4
✓	10ℓ	21	
✓	5ℓ	22	
✓	2ℓ	dNTPs	
✓	10ℓ	Pfu buffer	
✓	70ℓ	H_2O	
	1ℓ	Pfu	
	100ℓ		

30 cycles @ 55°

Add thymidine to proliferation assay.

result from PCR #23

Hafner 13/14 | PCR 22/23

- upper band is probably the correct product, cut out and reamplify

Continued on Page

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S. [redacted] Signed [redacted] Date

Marie Harrell Signed [redacted] Date

Day 3 staining T68515 for CD25

1) CD4 + iso TC
2) + CD25 TC

745
1171
1190
10) 1200

} UGFR FITC CD4 PE

New buffycoat V15721

cell # 1) 156 x 2 x 50 = 15600 x 10^4 = 1.5×10^8
2) 124 x = 1.24×10^8

150 µl α-CD4 bio per tube
500 µl beads -> activate

PCR # 24 :

✓	10 µl	PCR 23 upper band
✓	1 µl	EGFR1
✓	1 µl	4
✓	10 µl	Phu buffer
✓	2 µl	dNTPs
	1 µl	Phu
✓	75 µl	H_2O
	100 µl	

30 cycles @ 60°C

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Strategene #24 PCR prod.

Cut upper band
-? gel isolate

after gel extraction

2 µl

PCR # should be 307 b

Cut with BamHI / NheI in buffer H

and pCineo EGFR Δ

use all of gel elution

28 µl reaction
4 buffer H V
2µl BamHI
2µl NheI 18µl MQ V
40µl @ 37°C o/n

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PCR #24

Continue cloning of EGFRvIII xC into pCineo EGFRvIII xC

1) ✓ 3 µl plasmid (= 3 µg)
✓ 1 µl Nhe I
1 µl Bam HI
✓ 2 buffer H
✓ 13 µl H_2O
20 µl

CIP same as #3

Cloning of Flag/Geba into pCineo Geba

2) ✓ 3 µl Flag/Geba (#104)
✓ 1 µl Nhe I
✓ 1 µl buffer H
✓ 5 µl H_2O
10 µl

2 hrs @ 37°C

✓ + 1.5 µl buffer H
1 µl Pst I
✓ 7.5 µl H_2O
20 µl

3) ✓ 1 µg pCineo Geba
1 µl Nhe I
✓ 1 µl buffer H
✓ 8 µl H_2O
10 µl

✓ 4
✓ 5
✓ 1
20

+ 8 µl CIP buffer
6 µl CIP
44 µl H_2O

Continued on Page

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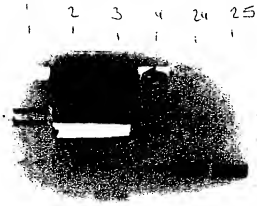
Signed [redacted] Date

Signed Date

PCR repeat PCR #24 V V 10µl #23
1µl EGFR1
V V 1µl EGFR4
V V 10µl Phu buffer @ 62°C
V V 2µl dNTPs 30 cycles
1µl Phu
V V 75µl H2O
100

#25 V 20µl #21
V 5µl #22
1µl EGFR1
V 1µl 4
V 10µl Phu b
V 2µl dNTPs
1µl Phu
V 60µl H2O
100



agarose a doublet shows up

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Cut PCR 24 and 25 with NheI / BamHI [redacted]

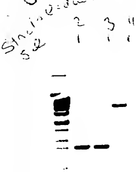
40l #24	40μl #25
5l buffer A	5l buffer A
2.5l NheI	2.5l NheI
2.5l BamHI	2.5l BamHI
50l	50l

~~Blunt BamHI site in pCRScript that contains EGFR∆IC~~ [redacted]

~~1l pCRScript EGFR∆IC~~
~~2l buffer B~~
~~2l BamHI~~
~~15l H2O~~
~~20l 1hr @ 37°C~~

Reamplify #24 and clone blunt end into pCR Script @ 62°C → #26

gel isolate 2, 3 and 4



1)	2)	3)
√ 2l #2	√ 2l #3	
2l #4	2l #4	2μl #4
√ 4l H2O	√ 4l H2O	√ 6l H2O
2l 5x buffer	2l 5x buffer	2l 5x buffer
10l		
+ 10l 2x buffer		
+ 2l ligase		

didn't get any clones =)

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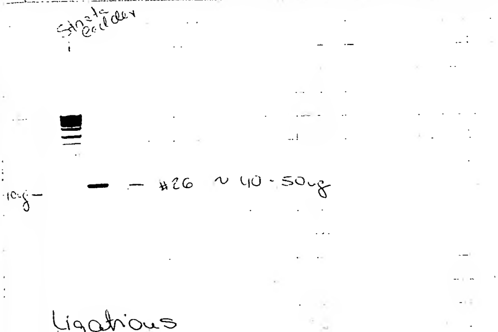
B. Bigg [signature]
Signed

[redacted]
Date

Signed

Date



Ligations

I ✓ 1l PCR Script
✓ 1.5l #26 (EGFR XCA)
✓ 1l PCR Script reaction buffer
✓ 0.5l ATP
✓ 4l H_2O
1l SrfI
1l T4 DNA ligase
10l

II ✓ 1l CIP
✓ 3l #2
✓ 4l H_2O
2l 5x buffer
10l 2x buffer
1l ligase

III ✓ 1l CIP
3l #3
✓ "
"
"
"

IV ✓ 1l CIP
✓ 7l H_2O
"
"
"

V ✓ 1l SAP
✓ 3l #2
✓ 4l H_2O
2l 5x buffer
10l 2x b
1l ligas

VI ✓ 1l SAP
3l #3
✓ "
"
"

VII ✓ 1l SAP
✓ 7 "
"
"

Continued on Page

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PROJECT Generation of EGFR ΔXC — Notebook No. 1510

Continued From Page 9

-> still didn't get any colonies after transformation

use less ligation, instead of 5 (for I) or 10l (II-VII) only 2l (works better according to Ross)

retransform #I, V, VII

-> also cut pCineo GabaR and minipreps 7 and 8 again with NheI and PstI, ligate, but no cipping or sapping

1) ✓ 2l pCineo GabaR
✓ 2l buffer H
2l NheI
✓ 14l H_2O
20l

2) ✓ 5l mini #7
✓ 2l buffer H
"
✓ "

3) ✓ 5μl mini #2
✓ "
"
✓ "

2hrs @ 37°C

+ 3l buffer H
2l PstI
15l H_2O
40l

run and cut out bands

Continued on Page

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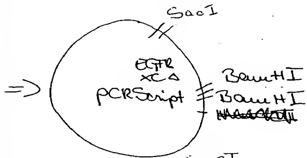
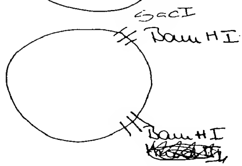
PROJECT Generation of EGFR ΔxC   Notebook No. 1510
Continued From Page 10

⇒ got about 200 clones for [redacted]
ligation #1 after the transformation
⇒ less ligation seems to work better

Let Peter check minis with Bam HI alone and
Bam HI + ~~Hind III~~ SacI



Bam HI ~~8150~~ vector linearized
+ ~~Hind III~~ SacI 350 + 3kb



Bam HI 350 + 3kb
B + S "

Set up new ELISA for IFN-γ day 1:

20 l as starting point: ~~248 l dilution buffer + 6 l~~

400 l dil. b
+ 100 l sup.) 250
250 dil. b.   4 dilutions

set up plate ~~same as for IL-2 etc~~
see next page

Continued on Page

Signed: S. Rigg   Date: [redacted]
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Signed: [signature]   Date: [redacted]

Test various EGFR antibodies

Transfect 293T with wt EGFR(100), EGFRΔIC(81, EGFRΔXCΔIC(121)

- seed 8×10^6 cells the day before transfection

100 : 1.18 μg	12.7 μl	+ 75 μl $CaCl_2$	+ 512 H_2O	= 600
81 : 1 μg/μl	=> 15 μl		510	= 600
121 : 1.15 μg	13 μl		512	= 4

+ 600 2× HBS

according to Janet's protocol

Isolation DNA from Pichia clones

followed Invitrogen protocol

- used lyticase instead of Zymolyase, didn't have enough units and incubated the mixture @ 37°C instead of 30°C
  => then left mixture o/n at 30°C

To make probe: 10 μg pPICZαB MusK # 30
≈ 10 μl
2.5 μl EcoRI
2.5 μl ClaI
5 μl buffer H
30 μl H_2O
50 μl

Continued on Page

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Date

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PROJECT pSecTagMusk | Test of EGTR ab in COS-1 Notebook No. 1510

Continued From Page 16

finished prep. of yeast DNA [redacted]

thaw Cos-1 cells

New transfection of 293 cells [redacted]

- this I plated ~ 3×10^6 day before transfection

- transfection itself was done as on [redacted]

mock: 131 $\times 20 \times 10^4 = 2620 \times 10^4 = 2.6 \times 10^7$ 350 ↓

100 : 126

81 : 123

121 : 184

staining:

1)	#100	PI	
2)		Ig2a	
3)		Ig2b	
4)		EGFR-PE	
5	mock	α EGFR PE	1
6		GR01	2
7		GR05	3
8	100	1	
9		2	
10		3	
11	81	1	
12		2	
13		3	
14	121	1	
15		2	
16		3	

FACS # are different

Continued on Page

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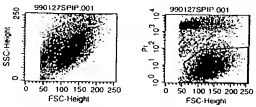
Test EGFR Antibodies

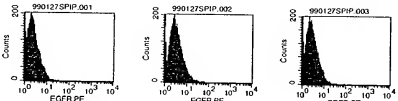
Notebook No. 1510

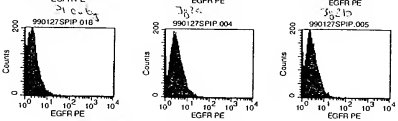
Continued From Page 17

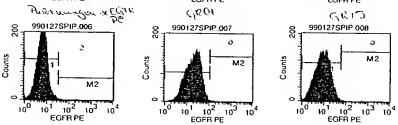
PROJECT

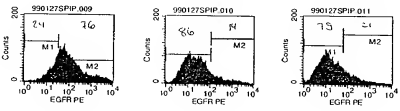
Results from p. 17

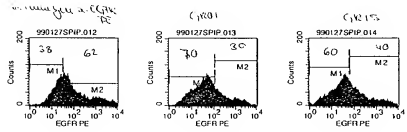


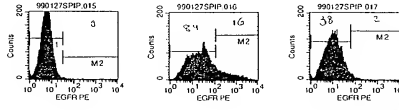












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Date

Purpose clone hEGFR ΔEC

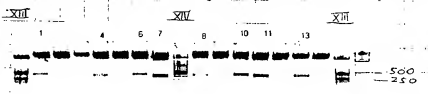
A. pCRScript-hEGFRΔEC #118

Suzanne has lgt hEGFRΔEC into pCRScript. Need to check w/ BamHI + Sac I

[redacted] innoculate 3 clones from [redacted] & 12 clones from [redacted] (# 4-14) → 3 ml

Bio 101 RPM mini-prep w/ 2 ml O/N cultures

	mini
	8
A	2
BamHI (10U/λ)	1.75
Sac I (40U/λ)	025
dd H2O	8
	20 λ

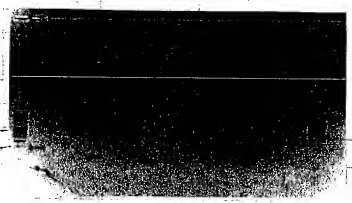


37°C 2 hrs

↓ run longer

2% TEA agarose

← gel "smiling"



#1, 4, 6, 7, 8, 10, 11, 13 show expected fragment sizes of 350, 3000 bp

Innoculate #1 (≡ 118.1) for maxi

6 (≡ 118.6)

Send 118.1-118.6 for sequencing

B. pG1a-hEGFRΔEC

pG1a

Not + Xho I

→ [illegible]

See pg 11 for RE analysis & td & FACS

Continued on Page 8

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Signed [redacted] Date [redacted] Signed [redacted] Date [redacted]

c. To clone hEGFRΔEC into hEGFRΔ

#81		#119.1	
pGla-hEGFRΔ	20	pGla-hEGFRΔ	20
B	5	B	5
BamHI	5	BamHI	5
ddH2O	20	ddH2O	20
	50		50

37°C 3hrs
expect 885 5880 bp
37°C 3hr
expect 18. 5005 bp

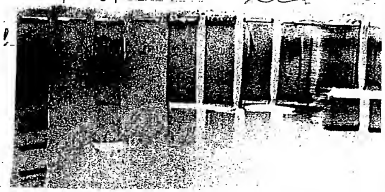
#83		#118.1	
pCRSu-hEGFR	20	pCRSu-hEGFR	
BamHI	5	BamHI	
EcoRS	5	RS	
B	10	B	
ddH2O	60	ddH2O	
	100		

37°C 3hrs
expect 2127, 2976
37°C 3
expect 18.

dephos #119.1 & #118.1 ⇒ 50 λ RE
10 λ CIP buffer
10 λ CIP
30 λ
100 37°C 2hr

gel isolate

BamHI	BamHI +RS	BamHI +RS	BamH
81	83	1181.1	1191.

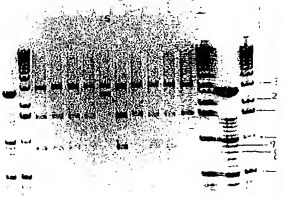


83 seems weird ∴ abandon cloning pCRSu-hEGFRΔ into pCRSu-hEGFRΔEC !

(λ)	I	V
ligt.		
119 ϕ	1	0
119+81 (½)	1	4
119+81	1	7

25°C ~~20°C~~ 20'
+fm XL 10 gold
leave on bench at 25°C over the weekend. Innoculate

pGla-hEGFRΔEC-hEGFRΔ
1 2 3 4 5 6 7 8 9 10



Mini	4			
B	1	correct orientation	opposite	No insert
EcoRS	1	75	75	75
ddH2O	4	76	76	76
	10λ	231	231	231
37°C 1hr		3016	3016	3016
load all		32	917	75
		2460 bp	1575 bp	
		#5	#1-4, 7-10	none

1% TCA 1hr @ = 3V

Continued Page

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Signed Date Signed Date

PROJECT hEGFR ΔEC 4

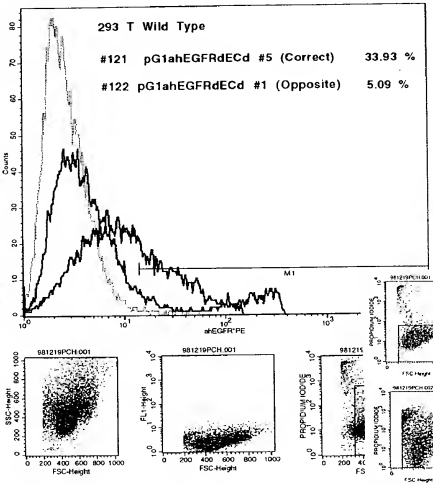
Inoculate #5 & 1 for maxiprep
Seed 293 Ts for transfection
Transfect 30 µg DNA } w/ Clontech's CaPO4 kit
15 µg pCIGH } → 600 λ w/ → add drop by drop 600 λ HEPES
15 µg pCIGP } DI H_2O while vortexing
74 λ $CaCl_2$ 25°C 25'
Add 50 λ 10 mM chloroquine to 10 mL in room
Add 1.2 mL mixture drop by drop
37°C 8 PM ~

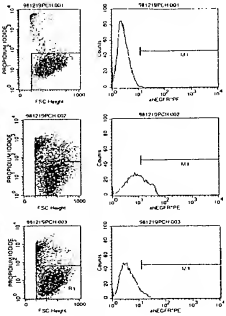
change fresh media ~ 9AM

FACS ~ 10 AM ∴ 36 hrs
post-transfection
collect sups at 36 & 60 hrs.

Future action:
① Td PPA6's
② Sort PPA6's

Overlay of 293T's Transfected with human EGFR Extracellular Deletion Mutant





Read and Understood By

Peter Clancy
Signed

Date

Signed

Date